The Effects of Ethanol on the Development of Brain Area in Chick Embryos

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I. Introduction

Fetal alcohol syndrome is prevalent in the United States. It affects between 3 and 22 live births out of every 10,000 (Center for Disease Control and Prevention, 2004). Fetal alcohol syndrome is described as a cluster or pattern of related problems. There are many signs of fetal alcohol syndrome such as small head circumference and brain size, small eyelid openings, sunken nasal bridge, an exceptionally thin upper lip, heart defects, deformities of joints, limbs and fingers, slow physical growth before and after birth, vision problems, delayed development, abnormal behavior such as short attention span, hyperactivity, poor impulse control, extreme nervousness and anxiety (Center for Disease Control and Prevention, 2004). Alcohol exposure at early embryo stages causes cell death which can lead some of the above abnormalities (Smith, 1997).

In a study conducted by Swanson, Douglas J. et al daily injections of approximately 30 mg/day of ethanol either chronically from embryonic days 4 to 15, or acutely from embryonic days 15 to 18 were administered. Untreated embryos were injected with saline and served as controls. They found that chronic ethanol reduces the weight of several brain regions (Swanson, 1994).

The developmental process that I studied was the brain and its development. I studied the size of the brain of chick embryos that have been subjected to levels of alcohol compared to normally developing chick embryo brains. In this study I tested the hypothesis that the brains of chick embryos that have been exposed to greater concentrations of ethanol will develop at a slower rate and eventually be smaller than those of the embryos exposed to a lesser concentration. I also hypothesize that exposing an embryo to any concentration of ethanol will result in the embryo’s brain being smaller and developing at a slower rate than the brain of the control embryos.

In chick embryos the brain is large and visible after just a few days of incubation. This makes it easy to locate and measure. Chick embryos are a good organism to study since they have very similar developmental pathways as humans and can be compared easily. Since research about chick development can be compared to humans, by observing chick brains and their size we can begin to understand more about human brain development. Most researchers who are looking to find out more about how fetal alcohol syndrome affects humans, study its effects on chicks and mice. Mice embryos are not as easy to obtain so in this experiment the best organism is the chick. In addition the chick embryo is separated from maternal interactions unlike the mammalian fetus and may be even more useful in studying the mechanisms by which ethanol directly influences brain development (Swanson, 1994).

In this study I explanted chick embryos and observed the growth of the brain. To conduct this study I performed an experiment where I placed my explanted embryos in weigh boats with three different concentrations of ethanol on day two of development, two embryos in a control of 0% ethanol, two embryos were exposed to .002% ethanol, and the remaining two were exposed to .02% ethanol. Each embryo also was given a concentration of penicillin/streptomycin

solution. I observed the embryos for six days or until their death by taking pictures of the brain. Later I changed the contrast on the images and measured the area of the brain in pixels and then converted square pixels to square millimeters with computer imaging programs.

II. Materials and Methods

Materials and equipment:

- Dissecting microscope SMZ 660 with camera DC200
- 8 fertilized eggs
- 8 sterilized weigh boats
- sterilized forceps
- Styrofoam bowl
- 70% ethyl alcohol
- Room with fume hood
- humidified incubator
- computer with digital imaging software, BTV Pro, Adobe Photoshop and Image J
- printer
- penicillin in streptomycin
- pipette
- 11mL Tyrodes solution
- 0.55mL ethanol

Methods

Explanting the embryos

Eight fertilized eggs that were incubating for two days, a pair of forceps (cleaned with 70% alcohol), a paper plate, and eight weigh boats were obtained. Weigh boats were prepared by folding up all four corners. All equipment was sterilized. The weigh boats were sterilized under the hood and placed in petri dishes with covers before being transferred to the ICUC where the explanting took place. The bench top, our hands, and eggs were all sterilized with 70% ethyl alcohol.

Explanting then began; the egg was held so that the wide end was facing up, with the embryo facing the egg's narrow end, away from the air space. The top of the egg was cracked very gently, while being careful not to push the shell into the egg. The tips of the forceps were used to remove small pieces of the shell at a time and expose the air-space. The hole was made as large as the air space, and the edges were made as smooth as possible to help ensure that the yolk was not torn as it came out the hole later. All this work was done over a Styrofoam bowl. A small slit was then made in the egg shell membrane, and the egg was carefully flipped over so the hole was almost touching the bottom of the inside of the weigh boat. A small hole was then made with the tip of the forceps in the narrow end of the egg. Air was then allowed in and the contents of the egg flowed out the large hole in the bottom into the weigh boat.

We made sure that the embryo was on top. If it wasn't we turned the egg yolk slightly using the blunt end of the forceps, but we had to be careful. Once explanted the embryo was covered with a petri dish cover at all times unless we were adding a solution to the embryo. The embryos were placed in the incubator in the ICUC when it was going to be a long time before we were able to add the penicillin/ streptomycin mixture or the Tyrodes salt solution. We added .5mL penicillin/ streptomycin mixture to each embryo. This mixture comes from a 10,000U stock solution that had 10mg streptomycin per milliliter; we needed .5mL of penicillin, we had 5,000units/ 5mg which is .1mg or 100 micrograms. So we had 100units of penicillin per 100 micrograms or streptomycin per 1mL. This procedure was repeated for each egg.

We started with eight embryos but we cracked two of the yolks both weeks so we only used six of the embryos in our

experiment. We used 2% ethanol and .02% ethanol in this experiment. To come up with our 2% ethanol we added 0.5mL of ethanol to 5 mL Tyrodes salt solution, we then only used 1mL of this solution each week but enough was made up so that other experiments could use the same solution. To make our 0.2% ethanol we added 0.05mL of ethanol to 5mL of Tyrodes salt solution. Again we only used 1mL of this solution each week but other experiments required this concentration also so one stock solution was made up.

We added .5mL of just Tyrodes salt solution to two of the embryos, which were the control embryos. We also added .5mL 0.2% ethanol in Tyrodes solution to two embryos, which were the experimental embryos that were exposed to .002% ethanol because the ethanol was diluted further when it was added to the embryo. The remaining two embryos had .5mL 2% ethanol in Tyrodes solution added to them, which were the experimental embryos that were exposed to .02% ethanol. The control embryos were the embryos that only had the Tyrodes salt solution and the penicillin/streptomycin solutions added to them; these controls were used to compare the other concentrations of ethanol to what a normally developing embryo would look like (Armstrong, 1994).

We then placed the weigh boat covers over the weigh boats and placed the embryos in a humidified incubator. This incubator contained two trays of water that allowed the air in the incubator to be humid; this allowed the embryos to not lose water and dry out. This procedure took about two hours to complete.

Explanting took place on day 2, the following day which was the first day that data was collected was day 3, and following day was day 4 and so on.

Observing explanted embryos over seven days or until embryo death

Each day following explantation when observing the embryos, we removed the weigh boats from the humidified incubator across the hall and placed them under the dissecting microscope SMZ 660 with camera DC200 and observed under .8 magnification. On day 4 we observed the embryos under 1 magnification. We took pictures of embryo’s brain using the digital imaging software BTV Pro. Once all the embryos had been observed they were placed back in the humidified incubator, and we returned the next day. When an embryo died it was placed in a metal bowl outside of the incubator. Each day this procedure took about thirty minutes.

How to change the contrast on an image

The image was opened Adobe Photoshop. “Image” was clicked on, on the tool bar, and then adjustments, brightness/contrast were selected. To boost the contrast the curser was moved up the line. The same contrast of 40 was used on all the images. This altered picture was then saved. This procedure took about thirty minutes to conduct on all of the pictures that were analyzed.

How to find the area of the brain of a chick embryo

The image with adjusted contrast was opened in Image J. Polygon was selected which is the third tool on the toolbar. The area to be measured was selected. The measurements were started at the middle edge of the left hand side of the eye. The eye can be identified as a dark crescent shape that has an opening off one side of it. A straight line was drawn from the above point to the edge of the embryo head; this line sliced the embryo in a cross-sectional direction. The line ran parallel to the heart. Then it went around the top of the head, around the nose, and continued right above the heart. When the end of the top of the heart was reached a straight line was made from that point to the starting point. “Analyze” was clicked on the tool bar at the top, and “measure” was selected from the options in analyze. This gave the area in pixels. This measurement was taken three times on each embryo and an average was taken. One control embryo lived to day 6 and was able to be measured each day from day 3 to day 6; this embryo is the one whose brain was analyzed for the control data. Only one embryo that was exposed to .002% ethanol was alive and able to be measured on day 3 and none after that. Only one embryo that was exposed to .02% ethanol was alive and able to be measured on day 3 and none after that. For each day, in each group (control, .002% ethanol and .02% ethanol), only one embryo was used to collect data about the brain. Conducting this procedure on all of the embryos analyzed took three hours.

How many pixels are in one millimeter

A picture of a ruler was taken on a dissecting microscope SMZ 660 with camera DC200. This picture of a ruler was opened in Image J that was taken at the same magnification as the picture that was being converted from pixels to

millimeters. It was done for both .8 magnification and 1 magnification. The first tool in the toolbox which is "rectangular selections" was clicked on. The corner of one of the millimeter marks was clicked on and as the line was dragged out the shift key was held down; this allowed the area being measured to stay square. The box was started from the right hand side of one of the millimeter marks and went to the right hand side of the next millimeter mark. After the area was selected, "analyze" was selected on the toolbar and "measure" was selected from the options in analyze. This gave the number of square pixels in one square millimeter. The procedure was repeated three times and an average was taken of the trials. The number of pixels in the area of the brain that was measured before were then divided by the number of pixels in one square millimeter at the correct magnification. This gave the correct square millimeters in the brain of the embryo. This procedure took about an hour.

III. Results

Explanting chick embryos on day two and keeping them alive long enough to gather data is a difficult task. Our control embryos did live longer overall; but they all died before day 7 of incubation and most of them much earlier. During the first week of this study all of our experimental embryos either died right after explantation or seemed to have never been fertilized. We observed all four of these embryos for two days following explantation and in one of the embryos exposed to .002% ethanol and one of the embryos exposed to .02% ethanol we saw small red smudges. These red smudges showed us that an embryo had begun to form and then died. In the other two embryos there was no evidence of this blood, leading us to believe that these eggs were never fertilized. Both of the control embryos this week were difficult to observe. For one of the embryos the structure of the embryo and its brain were not visible until day 4. Also some days the vascularization was covering part of the brain, so I was unable to measure it.

This week taught us a lot about what we were looking for and how to measure parts of the embryos; we didn't use any of this week's data in our final analysis. Another observation we made about the vascularization was it is very helpful in determining if an embryo is alive or not. When an embryo is alive it has continuous vascularization that grows as the embryo does, when the embryo dies the vascularization becomes discontinuous and there are visible breaks in it. In addition to the vascularization change the heart also visibly stops beating and the embryo begins to become less translucent and more opaque.

During our second week of data collecting we had a larger number of surviving embryos. We successfully explanted six embryos and of those six embryos three grew and developed through to day 3 (one control, one exposed to .002% ethanol and one exposed to .02% ethanol). Two of these embryos grew and developed through day 4 (the control and the embryo exposed to .002% ethanol) and only the control lived through to day 6. Throughout the week all of our yolks broke except the one exposed to .02% ethanol which lived to day 4. The yolks breaking did not seem to affect the embryos' survival rate as much as I thought it would, healthy embryos with broken yolks continued to live for a day or even a couple of days depending on the embryo. There seemed to be no correlation between when the embryos died and when or whether it had a broken yolk.

To quantify what I was observing I measured the number of pixels in the brain of the embryo as described in the procedure. I then converted pixels to millimeters squared to find the area of the embryo's brain in millimeters squared. As a result of many of the embryos dying before they could be measured I had a very small sample size. The area of the brain of the single control embryo that lived through to day 6 is shown in the graph below.

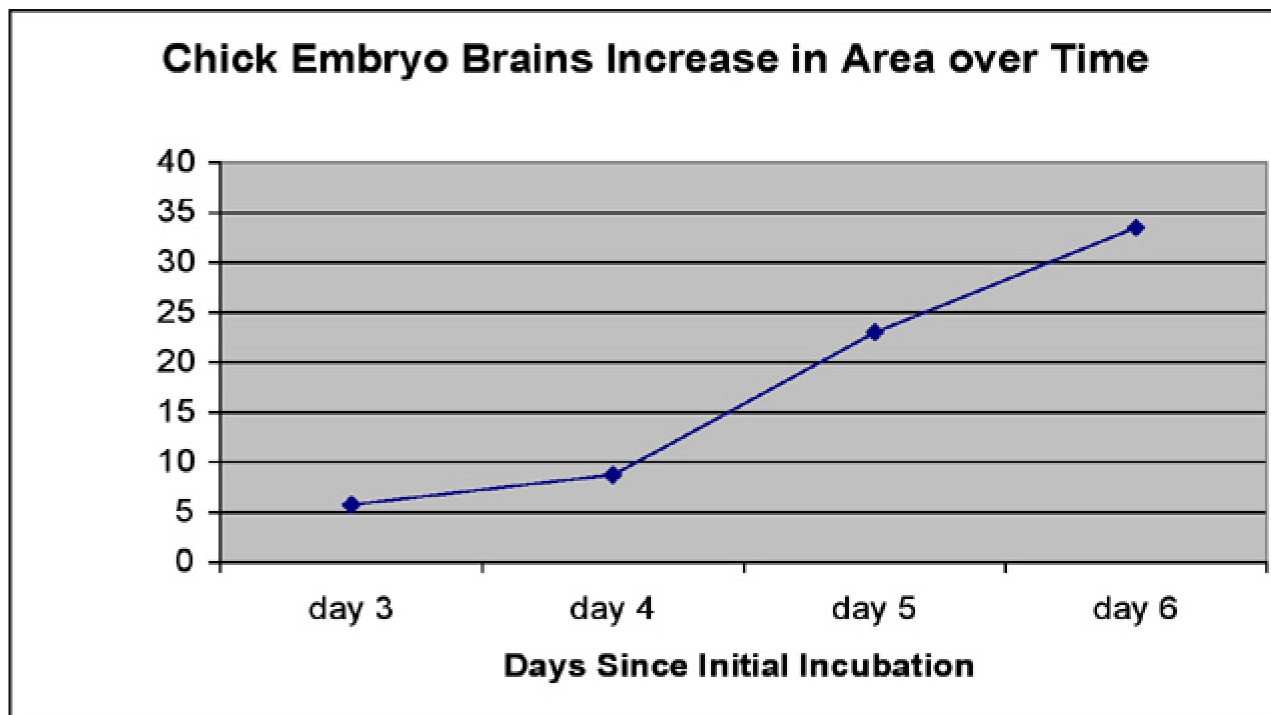

Figure #1: Chick Embryo Brains Increase in Area over Time. This graph shows the growth of the brain of a control embryo from day 3 to day 6 of development. This control embryo was not subjected to any ethanol. For all data points n= 1.

The control embryo's brain continued to increase in size from day 3 to day 6. As shown in figure #1 the brain appears to increase only by a small amount from day 3 to day 4, but then it increases in a more linear fashion from day 4 to day 6.

My pooled data from both weeks only contained one embryo that had been exposed to .002% ethanol that lived through to day 3 that I was able to measure the brain on. I also only had one embryo that had been exposed to .02% ethanol that lived through to day 3 that I was able to measure the brain on. This embryo actually lived to day 4 but on day 4 I was unable to see the whole area of the brain. Part of the vascularization had folded over and was covering part of the embryo's brain, and I was unable to see the part of the brain, making it impossible to measure. This embryo's yolk was very damaged. Having a damaged yolk may have caused the vascularization to not grow properly thus folding over; the embryo died before the next day of observations. Figure #2 is of a picture taken on day 4 of the embryo exposed to .02% ethanol; this picture has also been altered with a contrast of 40 to make it easier to see.

Figure #2 shows the embryo which was exposed to .02% ethanol, on day 4. This picture shows the vascularized area covering part of the embryo's brain making it impossible to measure the area.

The graph below compares the area of three chick embryo brains on day 3, after 24 hours of developing in the presence of; 0% ethanol, .002% ethanol or .02% ethanol.

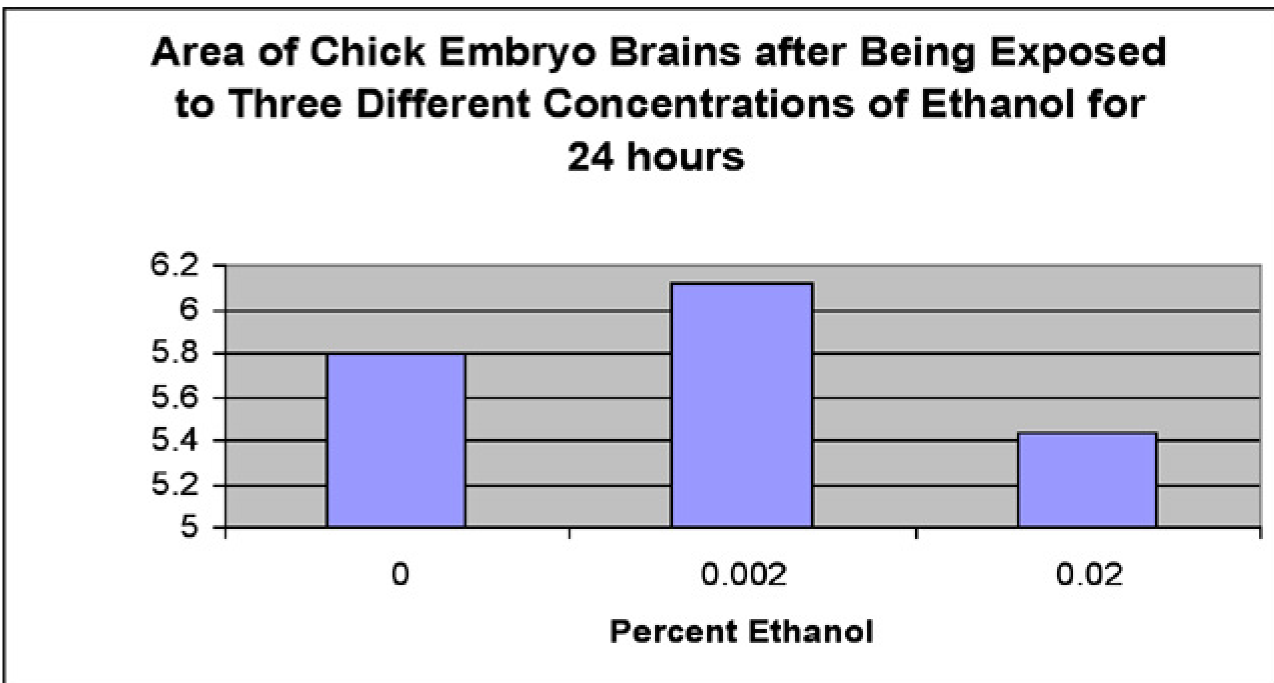

Figure #3: Area of Chick Embryo Brains after Being Exposed to Three Different Concentrations of Ethanol for 24 Hours. For all data n=1

Below is a picture of what a day 3 embryo's brain that was exposed to .002% ethanol looks like followed by a picture of what a day 6 control embryo's brain looks like.

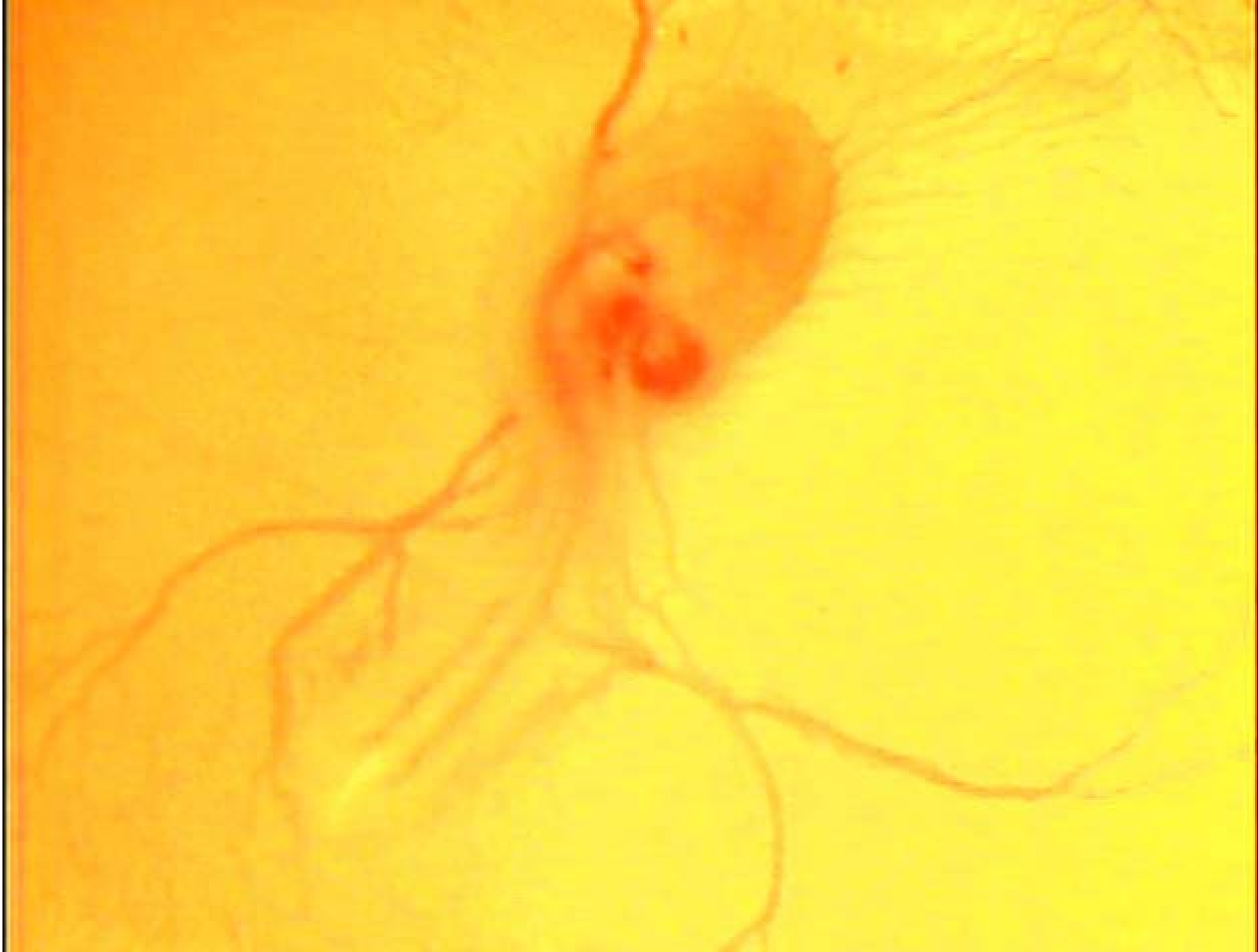

Figure #4 shows a day 3 chick embryo that has been exposed to .002% ethanol for 24 hours. This images contrast has been boosted to 40. The heart and optic cup along with the brain are all visible.

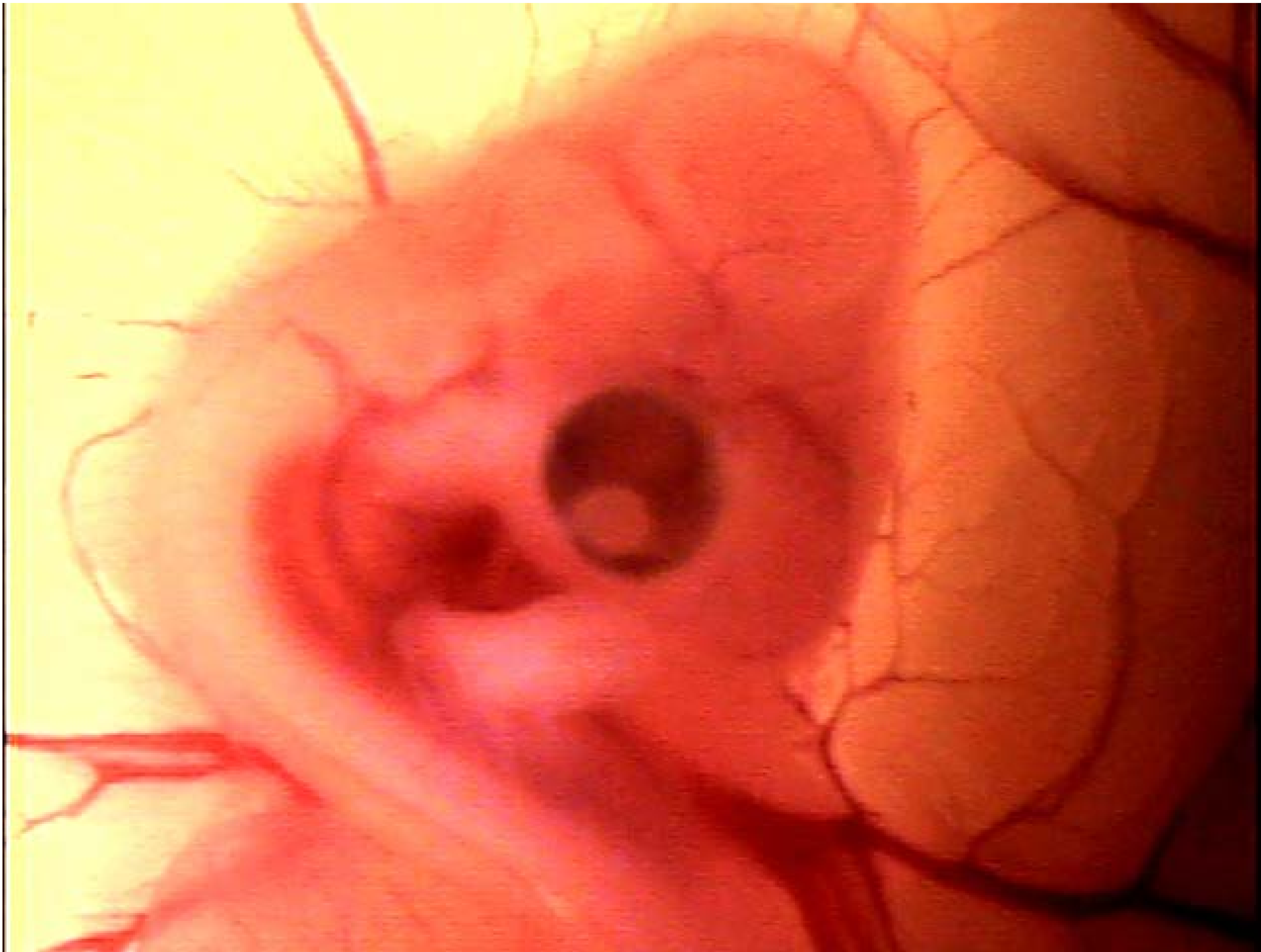

Figure #5 shows a day 6 control chick embryo that has not been exposed to any ethanol. The heart, optic cup and limb bud are all visible as well as the different regions of the brain. This image’s contrast has been boosted to 40.

IV. Discussion and Conclusions

From this study my hypothesis was neither supported nor refuted. From this data I can not say that the brains of the embryos that were exposed to ethanol will develop at a slower rate and eventually be smaller than those of the control embryos; or that the brains of the embryos exposed to a greater concentration of ethanol will develop at a slower rate and be smaller than those of the embryos exposed to a lesser concentration because I had such a small sample of data. I can conclude that the area of a normal chick embryo that is not exposed to ethanol grows and increases in size from day 3 to day 6. The rate of growth increased at a slow rate from day 3 to day 4, then from day 4 to day 6 the area of the brain seemed to be growing at a faster linear rate. One explanation for this may be that when the embryo is small and just beginning to grow, the brain is not the main focus of the growth. From day 3 to day 4 the embryo is focusing on increasing its length and developing its heart, and after day 4 many of the vital structures are formed and the embryo can begin to work on expanding the area of the brain. From day 4 to day 6 the brain area increases in a linear fashion. I don’t have any data for the embryo after day 6 because it died before day 7 but I would guess based on my data that the area of the embryo’s brain would continue to grow linearly.

My data also shows that the area of the brain of a chick embryo that was exposed to .002% ethanol is larger on day 3 then the area of a control chick embryo. One explanation for this could be that the embryo that was exposed to the ethanol was larger to begin with then the embryo that was kept as the control environment. In the two days that these embryos had to grow and develop before one of them was exposed to ethanol, the one that was exposed to ethanol grew more. I was not able to measure anything about the embryos before they were exposed to ethanol on day 2 because they were not developed enough, so I could not determine if all the embryos were the same size to begin with. If the embryo

that was exposed to .002% ethanol on day 2 was larger than the control embryo then the ethanol may have affected its growth, it may have slowed it, but it still ended up having a brain with a larger area on day 3 because it was larger to begin with.

Another explanation for why the embryo that was exposed to the .002% ethanol had a larger brain on day 3 could be because .002% ethanol is not strong enough to effect the embryo's brain's growth. This second reason is not as probable because this one embryo that I analyzed was the only embryo exposed to .002% ethanol that lived to day 3 and it died before day 4. I can also compare the embryo that was exposed to .02% ethanol to the control embryo and the embryo exposed to .002% ethanol. The embryo exposed to .02% ethanol did have a smaller brain area than both the control and the embryo exposed to .002% ethanol. All of this data can be seen in Figure #3. This data does support my hypothesis except again I was only looking at one embryo and this specific embryo may have been smaller to begin with then the other two before the ethanol was added. Because I have such a small sample, I only compared one embryo to one embryo to one embryo I do not have enough data to make any supported conclusions about the effect of ethanol on the brain.

One of my sources of error is in the fact that not all the embryos were necessarily the same size when the study first started and some of the embryos were exposed to ethanol. Another source of error is in measuring the area of the brain. I measured the same way on all the embryos and I measured each one three times and took an average but it is hard to tell sometimes exactly where one part of the embryo ends and another starts, even with the added contrast. I never got the same number of pixels twice which further proves that to follow the same path more than once is very difficult if not impossible especially when you are dealing with pixels. I tried to minimize the amount of error by measuring each embryo three times, but there is still a margin of error.

To refine this experiment I would use more embryos so that I would hopefully end up with more surviving embryos to analyze. By having more embryos I would minimize the amount of error based on different sizes of embryos before the study begins. I would also minimize the amount of error based on measuring the area of the brain. More embryos means more measurements and the more measurements I have to average the closer I will get to the actual value. For future experiments that should be done to extend my results would be just conducting the same study again with more embryos and collecting more data. More embryos I would be able to make a stronger and more supported conclusion.

V. Bibliography

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I worked in collaboration with Natalie Shelton on this study